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RAPID COMMUNICATION

The Interferon-Inducible 204 Gene, a Member of the Ifi 200 Family, Is Not Involved in the Antiviral State Induction by IFN-α, but Is Required by the Mouse Cytomegalovirus for Its Replication

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To examine whether Ifi 200 genes are involved in antiviral state induction by IFNs we expressed mutant forms capable of inactivating the endogenous p204 and analyzed replication of both RNA and DNA viruses following IFN-α treatment. Inactivation of p204 does not impair replication of vesicular stomatitis virus, encephalomyocarditis virus, ectromelia virus, and herpes simplex virus 1 and does not alter an IFN-α induced antiviral state. By contrast, in cells lacking functional p204, mouse cytomegalovirus (MCMV) replication is strongly inhibited and is not further modulated by IFN-α. These results suggest that p204, a member of the Ifi 200 gene family, is not involved in the IFN-α-induced antiviral activity against some RNA or DNA viruses, but is required by MCMV for its replication.

IFN binding to specific cell surface receptors activates a family of transcription factors termed signal transducers and activators of transcription, or STATs (*26*), that reside outside the nucleus in untreated cells, but become activated, multimerize, and translocate into it upon stimulation with IFNs. Here, by recognizing discrete *cis*-acting regulatory DNA sequences they stimulate transcription of so-called IFN-inducible genes or ISGs (*7*). The ensuing transient antiviral state is thought to result from the concerted action of a large number of IFN-induced proteins. Defining the relative contributions of these proteins is therefore a formidable task. To date, the physiological roles of only a few IFN-induced proteins have been resolved (e.g., Mx, PKR, 2′-5′ oligoadenylate-synthetase) (*16, 26*).

One family, designated Ifi 200, includes the p202, p203, p204, and D3 proteins (*20*) and their human homologues, MNDA (myeloid nuclear differentiation antigen) (*2*), Ifi 16 (*27*), and AIM2 (*8*). It is encoded by a cluster of six or more structurally related murine genes in the q21–q23 region of chromosome 1 (gene 200 cluster) and appears to be transcriptionally activated by IFNs both *in vitro* and *in vivo* (*17, 20*). These proteins share one or two partially conserved 200-amino-acid segments, designated domain *a* and domain *b*, respectively, and at least four of them are nuclear.

The 202 gene, the best defined member of the murine family, encodes a 52-kDa phosphoprotein that increases 20- to 30-fold in cultured cells in response to IFN-α and translocates into the nucleus after a few hours. Here, p202 binds to both pRB and the transcription factors E2F, p53, AP-1, c-Fos, c-Jun, and NFkB and inhibits their activity (*4–6, 22*). When p202 was stably transfected in cells, the 202 transfectants either expressed low p202 levels or lost the transgene after some time, indicating that cells do not tolerate high levels of p202 expression (*18*).

p204 is a 72-kDa phosphoprotein that, like p202, increases several-fold upon treatment with IFN-α and then translocates into the nucleus (*19*). The 204 protein functions as a growth suppressor in sensitive cell lines, as determined by cell focus assays. Transient p204 overexpression through a heavy-metal-inducible promoter in mouse embryo fibroblasts (MEF) lacking the endogenous p204 arrests cell growth by accumulating cells at the G1/S border (*19*). The 204 protein contains two LXCXE motifs that are potential sites for binding to the retinoblastoma gene product pRB. One motif is in the first 200-amino-acid domain at position 423–427, and the other is in the second domain at position 611–615. Recent results from our laboratory indicate that both motifs are required for p204 antiproliferative activity (unpublished observations). Moreover, *in vivo* studies by West-

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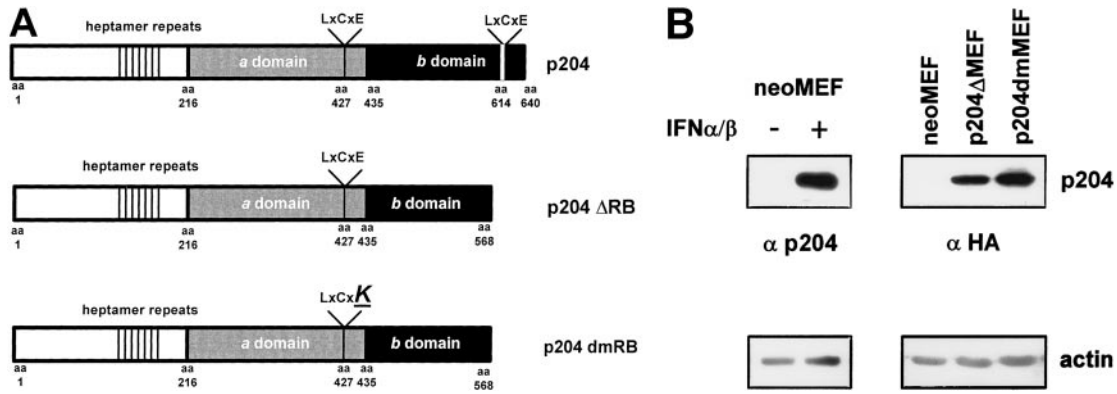

FIG. 1. (A) Schematic diagram of wild-type and mutant 204 proteins. The conserved 200-amino-acid domain (type *a* and type *b* segment) and the RB binding motifs are indicated. The seven vertical bars at the N-terminus indicate the heptamer repeats. The C-terminal deletion mutant p204ΔRB (del 640-568) harbors only the LXCXE domain present in domain *a*; the protein encoded by the construct indicated as p204dmRB harbors both the C-terminal deletion up to amino acid 568 and the point mutation from glutamic acid to lysine at position 427; therefore the resulting protein lacks any functional RB binding domain. Both mutant proteins have been cloned in the pRcRSV expression vector, harboring the drug resistance (G418) marker, with an in-frame fusion at the C-terminus with the HA epitope, transfected in MEF3T3 cells, and the resulting pooled population of neomycin-resistant clones were named p204ΔMEF (for p204ΔRB transfection) and p204dmMEF (for p204dmRB transfection), respectively. Control cells transfected with the empty vector (pRcRSV) are indicated as neoMEF. (B) Western blot analysis of endogenous or mutants p204 protein in transfected MEFs. Equal amounts (50 μg) of total protein extracts were separated on an 8.5% SDS–PAGE gel and blotted onto a PVDF membrane. The presence of the endogenous 204 protein from neoMEF untreated or treated with IFN-α for 24 h was detected with an affinity-purified rabbit polyclonal antibody (left). The expression levels of p204 mutants were monitored with anti-HA monoclonal antibodies (right). A Western blot of α-actin was included as internal control.

ern blotting and immunohistochemistry analysis demonstrated that, like the human homologue Ifi 16, p204 is constitutively expressed in myeloid cells and selectively induced by the synthetic double-stranded RNA, poly(rI:rC), in monocyte/macrophage-like cells (*12*). From these observations a potential role of p204 in cell cycle control and terminal differentiation has been inferred. By contrast, the role of the Ifi 200 genes in the induction of antiviral state by IFNs is still an open question.

As a first attempt to verify whether p204 does play any role in the IFN-mediated antiviral state induction, MEFs were transfected with the expression vector pRcRSV harboring both a C-terminal deletion mutant and a point mutant form of the 204 cDNA. A schematic representation describing the proteins encoded by each plasmid is presented in Fig. 1A. MEF3T3 cells were chosen for transfection because they do express p204 at high levels upon IFN induction, but not p202, behaving as an excellent cellular system for monitoring the antiviral activity mediated by the 204 protein. The plasmids harboring either of the mutated 204 proteins, p204ΔRB or p204dmRB, together with the empty vector were transfected in MEF3T3 cells, and pooled populations of neomycin-resistant clones were established and designated p204ΔMEF for p204ΔRB and p204dmMEF for p204dmRB transfection. Expression of either transfected mutants or endogenous p204 was assessed by both Western blotting and immunofluorescence analysis. The mutant forms were detected by using anti-hemagglutinin (anti-HA) monoclonal antibodies, whereas the endogenous 204 protein was detected with a rabbit polyclonal antiserum raised against the C-terminal moiety, as previously described (*12*). Figure 1B shows that both mutated proteins are expressed at high levels. As expected, p204 levels were almost undetectable in the control cells, transfected with the empty vector and designated neoMEF, but sharply increased upon IFN-α treatment. IFN treatment did not modify the expression levels of the mutated p204 (data not shown).

Immunofluorescence staining analysis by confocal laser scanning microscopy showed in untreated neoMEF a diffuse nuclear and cytoplasmic p204 staining (Fig. 2a), whereas upon IFN-α treatment for 24 h staining was predominantly nuclear (Fig. 2b). The mutated 204 proteins showed a subcellular distribution similar to that of the endogenous protein (Figs. 2c and 2d), demonstrating that the introduced mutations do not interfere with their cellular localization.

To evaluate whether the mutant p204 forms had any dominant-negative effects, neoMEF and cells transfected with either of the p204 mutant forms, p204ΔMEF or p204dmMEF, were virus infected and assayed for antiviral state induction upon IFN treatment. The viruses tested were representative of four families as follows: *Picornaviridae* (encephalomyocarditis virus, EMCV); *Rhabdoviridae* (vesicular stomatitis virus, VSV serotype Indiana); α- and β-*Herpesviridae* (herpes simplex virus, HSV-1, and mouse cytomegalovirus, MCMV, respectively); *Poxviridae* (Ectromelia virus, Ectr.V). They were all cytopathic and, with the sole exception of MCMV, lysed susceptible MEF3T3 cells within 48 h. Titers of the samples taken at 24 h postinfection (p.i.) with VSV and EMCV, at 36 h p.i. with HSV-1 and Ectr.V, and at 144 h p.i. with MCMV are shown in Fig. 3. Following IFN treatment,

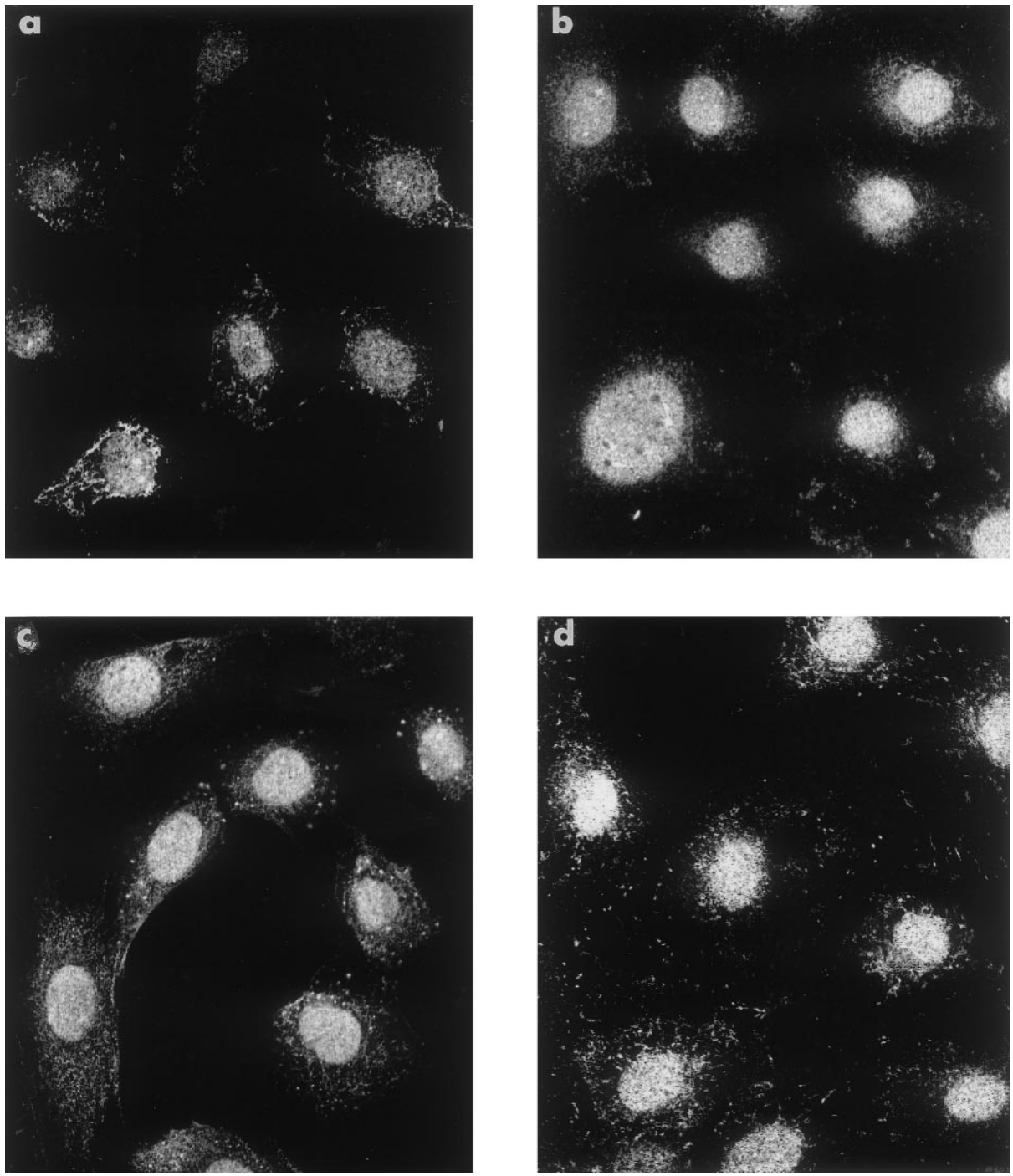

FIG. 2. Localization of endogenous or mutant p204 proteins in transfected MEFs by indirect immunofluorescence. NeoMEF, untreated (a) or treated with IFN-α for 24 h (b), was immunostained with affinity-purified anti-p204 rabbit antibodies; p204Δ or p204dm MEFs were immunostained with monoclonal antibodies against the HA peptide (c and d). FITC-conjugated anti-rabbit or mouse antibodies were used to detect the anti-p204 or anti-HA antibodies, respectively.

neoMEF showed a high degree of resistance to both RNA and DNA viruses: titers were indeed more than 100-fold lower than those of the untreated cells. These results indicate that neoMEF cells are permissive to all five viruses and fully responsive to IFN antiviral activity. A different picture emerged with p204ΔMEF and p204dmMEF cells. Both were fully susceptible before IFN treatment to RNA viruses, i.e., EMCV and VSV, and to two of the DNA viruses, i.e., HSV-1 and Ectromelia virus. As expected, a significant decrease in virus titer (more than 2 logs) was observed when the transfectants were treated with IFN-α, indicating that expression of the dominant-negative mutants did not interfere with its antiviral activity. By contrast, when untreated p204ΔMEF or

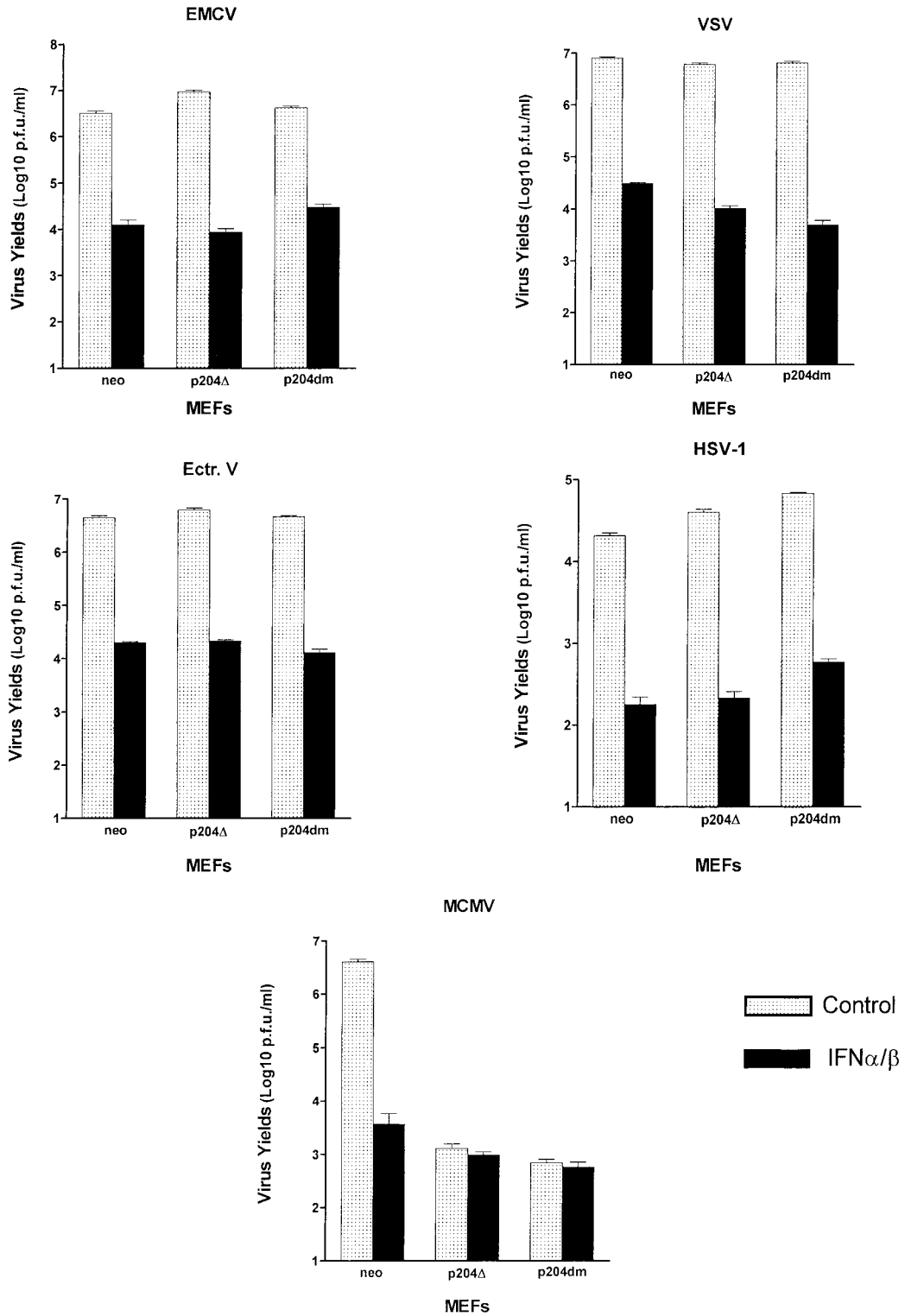

FIG. 3. Virus replication in neo, p204Δ, or p204dm MEF cells. Cells were infected with the following viruses: a rhabdovirus (VSV serotype Indiana), a picornavirus (EMCV), a poxvirus (Ectromelia, Ectr.V), an α-herpesvirus (HSV-1), a β-herpesvirus (CMV). m.o.i. ranging from 0.1 to 2 PFU/cell were used, but for simplicity only the results obtained with the m.o.i. of 1 PFU/cell are reported. The viral titers in the culture supernatants at the times indicated in the text for each virus were evaluated on Swiss MEF and virus yields are plotted.

p204dmMEF was infected with MCMV, a significant decrease in virus yield was observed (1.3×10^3 and 9.5×10^2 PFU, respectively) compared to that obtained with untreated neoMEF (4.1×10^6 PFU). When cells were treated with IFN-α no significant decrease in virus yield (8.9×10^2 PFU for p204ΔMEF and 6.4×10^2 PFU for

p204dmMEF) was observed, suggesting that inactivation of endogenous p204 impairs MCMV replication. Finally, kinetics experiments demonstrated that at later time points, i.e., 168 and 192 h, MCMV production from p204ΔMEF and p204dmMEF slightly increased (values ranging between 10^4 and 10^5 PFU) without reaching the levels of virus yields observed with neoMEFs at the same time points (values ranging between 10^6 and 10^7 PFU) (data not shown).

The lower MCMV yields observed in MEFs transfected with the dominant-negative mutants could be caused by decreased virus levels adsorbed by these cells at the early phases of infection. To rule out this possibility, the amounts of viral genome adsorbed were measured by a competitive PCR assay that allows the absolute quantification of small amounts of viral DNA by its coamplification with known amounts of added competitor DNA. The amplification target was a region of the MCMV IE1 gene spanning from exon 2 (5890 nt) to exon 4 (6529 nt), generating an amplification product of 639 bp (Fig. 4A) (*11*). A DNA fragment of the same sequence as the target viral DNA (except for an insertion in the middle to enable identification after gel electrophoresis) was used as competitor (*11*). As shown in Fig. 4B, as the number of competitor molecules added increases, the ratio between the upper and the lower band increases. Equivalence between genomic DNA from infected neo, p204Δ, and p204dm MEFs and competitor is obtained at the same concentration of 4×10^4 competitor DNA molecules, indicating that expression of the dominant-negative mutants does not affect the MCMV adsorption, but rather impairs subsequent phases of the infection.

IFN antiviral activity has been extensively studied: depending on the cell type and the virus, it affects viral penetration and uncoating, transcription, translation, and assembly of progeny viruses (*16*). The most convincing demonstration of the role of the IFN-inducible 2′-5′(A)synthetase/RNase L pathway in antiviral action comes from experiments in which expression vectors containing the cDNAs for the human 40-kDa isoenzyme or the mouse 43-kDa isoenzyme were transfected into different cell types. EMCV or mengo virus replicated poorly in these cells, whereas replication of VSV and HSV-2 was not affected (*3*). Protection of cells against human immunodeficiency virus (HIV) infection by the expression of 2′-5′(A) synthetase has also been reported (*24*). Another IFN-inducible protein, namely the protein kinase PKR, seems to be a central component in the antiviral resistance to picornaviruses and vaccinia virus. Replication of EMCV or vaccinia virus was indeed reduced in mouse 3T3 cells by expression of the cDNA encoding wild-type PKR, but not by expression of the PKR(1-551)K296R catalytic subdomain II point mutant, which lacks kinase activity (*10*). By far the most progress has been made with the Mx system as mediator of antiviral IFN activity (*25*). Swiss 3T3 cells transfected with a cDNA encoding the human MxA display a high degree of resistance to influenza virus and VSV, but not to two picornaviruses, to a togavirus, or to HSV-1. MxA protein interfered with intracytoplasmic transport of viral mRNAs, viral protein synthesis, and translocation of newly synthesized viral protein to the cell nucleus.

We have previously demonstrated that, like its human counterpart, MCMV up-regulates the cellular NF-κB activity needed for initial induction of the IE genes and progression of its replication a few hours after infection (*14*). By contrast, IFN-α inhibits MCMV IE gene enhancer activity by mechanisms that decrease the availability of virus-induced NF-κB transcriptionally active in the nuclei of infected cells (*13*).

The main conclusion from the experiments presented here is that expression of mutant 204 proteins devoid of one or both of the RB binding domains can interfere with the MCMV multiplication cycle but does not affect IFN antiviral activity. Our results clearly indicate that the IFN-inducible protein p204 is somehow related to MCMV replication. How MCMV exploits p204 is not known, though one model can be envisaged. It has been demonstrated that Ifi 200 genes are more related to control of the cell cycle than control of virus replication (*19*; unpublished results) and that HCMV infection arrests the cell cycle and alters the steady-state levels of several proteins involved in its regulation (*1, 9, 15, 21*). The present finding that inactivation of p204 by dominant-negative mutants deregulates the cell cycle and renders cells intrinsically resistant to MCMV replication might therefore suggest that MCMV exploits p204 to regulate the cell cycle and consequently its replication. Further support for this hypothesis comes from the finding that MCMV transcriptionally activates p204 expression a few hours after its adsorption to the infected cells and tightly regulates its levels of expression by posttranscriptional processes (unpublished observations).

Mouse embryo fibroblasts (MEF3T3) were kindly provided by T. Upton (Dana-Farber Cancer Institute) (*23*) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Gibco BRL). Where indicated, cells were treated for 24 h with 1000 U/ml of recombinant hybrid human IFN-α A/D (sp act 4×10^{-8} U/mg of protein), used as a source of murine IFN-α, since the antiviral activity is similar in human, bovine, feline, rat, and mouse cells (*28*). Transfections were carried out with Lipofectamine Plus (Gibco BRL) as specified by the manufacturer's protocol. Briefly, 3×10^5 MEF cells were transfected with 2 μg of the indicated plasmid and stable transfectants were selected by the addition of 800 μg/ml G418 (Gibco BRL), the medium being changed every 4–5 days until G418-resistant colonies began to appear (14–16 days after transfection). Pools of at least 100 colonies were harvested and maintained in G418 selection. Expression of the exogenous protein was eval-

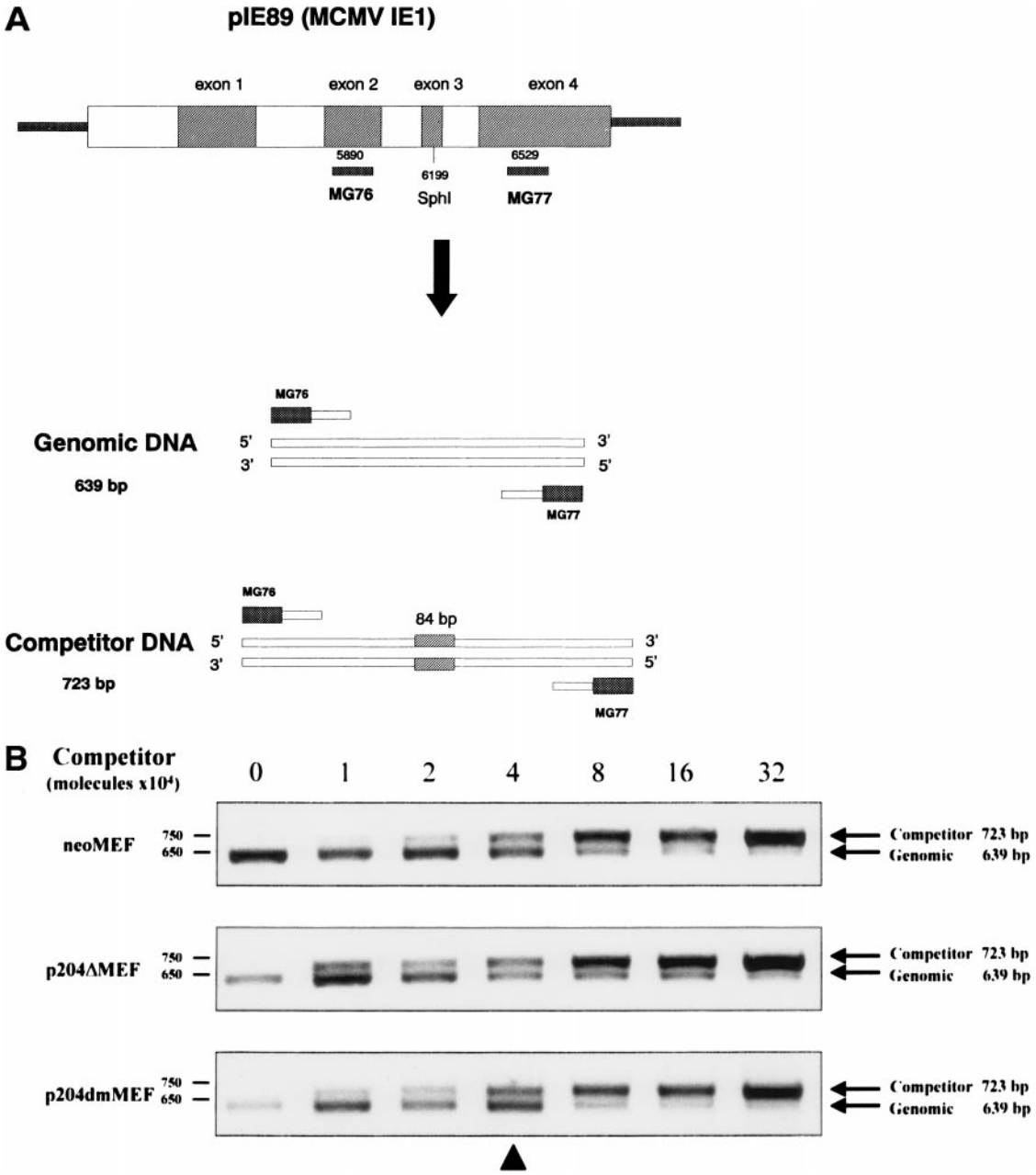

FIG. 4. Competitive PCR to quantify the MCMV particles adsorbed on neo, p204Δ, or p204dm MEFs. (A) Plasmid pIE89, derived from pACYC177, containing the IE-1 gene, was linearized with *Sph*I cutting exon 3 (6199) and an insert of 84 bp was cloned in this unique restriction site. MG76 and MG77 indicate the plus and minus primers, respectively (*11*). (B) Fixed amounts of genomic DNA (1 μg) derived from MEFs after 2 h of MCMV adsorption were amplified by 35 PCR cycles with increasing amounts (1–32 $\times$ 10^4 molecules) of competitor DNA for the IE-1 region. The upper and lower arrows indicate the amplification products for competitor and genomic DNA, respectively. Positions of the molecular mass markers (bp) are indicated on the left. The arrowhead on the bottom indicates the equivalence between genomic DNA and competitor. The bands of the amplification products corresponding to competitor and genomic DNA of the gel were quantified with Bio-Rad Analyst, Version 1.5.

uated by Western blotting using the anti-HA-probe monoclonal antibody (Boehringer).

The pRcRSV204 vector, which is tagged with a HA epitope, was constructed by PCR amplification (using *Pfu* DNA Polymerase, Stratagene) from pSVK3-204 containing the entire 204 coding region and cloning in the blunt-ended *Not*I site of pRcRSV (Invitrogen). Primers used to amplify the 204 cDNA were constructed in order to obtain a C-terminal fusion protein with the HA epitope as follows: N-terminal 5′-TCAcaggctggcatagtcagggacgtcataaggataGATGACCTGCATGTAACTGTGC-3′ (where TCA is a stop codon; the HA epitope is in lowercase letters); C-terminal 5′-TCAcaggctggcatagtcagggacgtcataaggataCTCTCCACTCACAAATGTCC-3′. The 204 RB binding site mutants were constructed by either oligonucleotide-directed mutagenesis or subcloning by stan-

dard procedure. To generate pRcRSV204ΔRB (for brevity p204ΔRB) (del 640–568), a 220-bp fragment from pRcRSV204 was removed by digestion with *Sca*I and religated. For the construction of pRcRSV204dmRB (for brevity p204dmRB), containing a point mutation in the Rb binding site at position 427 from E to K, the following two mutagenic oligonucleotides were utilized: MG144, 5′-GCAACCAAAGTTAGTGTGTGGA**aAA**CACAGTTTC-ATCAAGATATC-3′; MG145, 5-GATATCTTGATGAAACTGT-G**TTt**TCCACACA CTAACTTTGGTTGC-3′. The mutants generated are shown schematically in Fig. 1A. All mutations and in-frame junctions were confirmed by sequencing.

Total cell extracts were prepared by lysing cells in 3% SDS lysis buffer (*12*). Proteins were separated on a 8.5% SDS–polyacrylamide gel and transferred onto a PVDF membrane (Amersham), and Western blot analysis was performed as previously described (*12, 19*).

Neo, p204Δ, and p204dm MEF cells grown on coverslips were washed with PBS, fixed with 2% paraformaldehyde for 15 min at room temperature, and then washed again with PBS. The cells were subsequently permeabilized with 0.2% Triton X-100 for 20 min at 4°C, washed with PBS–1% BSA, and incubated with the anti-HA antibody (Boehringer) or the anti-p204 antiserum (*12*) in PBS with 10% DCS for 1 h at room temperature. After being washed with PBS–1% BSA, 0.05% Tween 20, the cells were incubated with FITC-conjugated anti-mouse or anti-rabbit secondary antibody in PBS–1% BSA for 1 h. Finally, coverslips washed with PBS were soaked with 1 μg/ml propidium iodide for 3 min, washed again with PBS, and mounted in 90% glycerol, 2.5 g/L diazabicyclo[2.2.2]octane (Sigma) in PBS. Immunofluorescence microscopy was performed on an Olympus IX70 inverted confocal laser scanning microscope, equipped with a krypton–argon ion laser (488/568 nm).

To assess the antiviral state, neo, p204Δ, and p204dm MEFs, untreated or treated with IFN-α (1×10^3 U/ml) for 24 h, were infected with EMCV, vesicular stomatitis virus (VSV serotype Indiana), Ectr.V, HSV-1, and MCMV, at m.o.i. ranging from 0.1 to 2 PFU/cell, but for simplicity only one m.o.i. (1 PFU/cell) is reported for each virus. The viral titers in the culture supernatants were measured on Swiss mouse embryo fibroblasts by the plaque-forming assay and are expressed as $\log_{10}$ PFU/ml.

Fixed amounts of genomic DNA (1 μg) extracted 2 h after virus adsorption from neo, p204Δ, and p204dm MEFs infected with MCMV at an m.o.i. of 0.1 PFU/cell were amplified by 35 PCR cycles with increasing amounts (from 1×10^4 to 3.2×10^5 molecules) of competitor DNA for the IE-1 region (*11*). The monolayers were extensively washed before DNA extraction to avoid co-amplification of DNA from viral particles nonspecifically associated with cells. The bands of the amplification products corresponding to competitor and genomic DNA of the gel were quantified with the Bio-Rad Analyst, Version 1.5.

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